

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
14 October 2004 (14.10.2004)

PCT

(10) International Publication Number  
**WO 2004/087920 A1**

(51) International Patent Classification<sup>7</sup>: C12N 15/11,  
A61K 48/00

AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,  
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,  
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,  
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,  
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,  
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,  
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM,  
ZW.

(21) International Application Number:  
PCT/IT2004/000117

(22) International Filing Date: 8 March 2004 (08.03.2004)

(25) Filing Language: Italian

(26) Publication Language: English

(30) Priority Data:  
RM2003 A 000149 2 April 2003 (02.04.2003) IT

(71) Applicant (for all designated States except US): GIULIANI S.p.A. [IT/IT]; Via Palagi, 2, I-20129 Milano (IT).

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(72) Inventor; and

Published:

(75) Inventor/Applicant (for US only): MONTELEONE, Giovanni [IT/IT]; Via Montpellier, 1, I-00133 Roma (IT).

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

(74) Agents: GITTO, Serena et al.; Ing. Barzanò & Zanardo Roma S.p.A., Via Piemonte, 26, I-00187 Roma (IT).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

WO 2004/087920 A1

(54) Title: ANTISENSE OLIGONUCLEOTIDES (ODN) AGAINST SMAD7 AND USES IN MEDICAL FIELD THEREOF

(57) Abstract: The invention relates to antisense oligonucleotidic sequences (ODN) against Smad7 suitably modified, and their uses in medical field as therapeutic biological agents, in particular in the treatment of chronic inflammatory bowel disease, such as Crohn's disease and ulcerative colitis.

~~Specs~~  
1020 Rec'd PCT/PTO 29 SEP 2005

Antisense oligonucleotides (ODN) against Smad7 and uses thereof in medical field

The present invention relates to antisense oligonucleotides (ODN) against Smad7 and uses thereof in medical field

Particularly the invention refers to Smad7 antisense ODN sequences suitably modified, which show a surprising biological activity of specific inhibition of Smad7 expression and are therefore usable in medical field as therapeutic biological agents, in particular in the treatment of chronic inflammatory bowel disease (IBD).

Crohn's disease (CD) and ulcerative colitis (UC) are the major forms of chronic inflammatory bowel disease in human. Both diseases are complex clinical entities, whose pathogenesis is strictly dependent on the interaction between different genetic, environmental and immune factors.

Despite CD and UC show marked differences both on the pathophysiological and clinical level, the therapeutic approach to suffering patients shares many common elements. Variability of the clinical presentation, of the type and the extension of the lesions, and of the kind of complications influences the therapeutic choice, even though the pharmacological treatment would represent the first predominant approach.

Salicylazosulfapyridine and 5-aminosalicylic acid are drugs of proven efficacy in the management of the mild form of IBD and in the remission maintenance therapy.

In the phases with moderate to severe activity and in the cases in which the general state is involved, it is necessary to turn to the use of corticosteroids. From the medium and long-term analysis of the main worldwide case histories, it appears that clinical remission is obtainable only in two thirds of patients receiving corticosteroids, and only in 50% of these patients it does not occur any relapse after drugs suspension.

The continuous administering of corticosteroids, beside inducing drugs dependence phenomenon, is worsened due to a very high risk of side effects.

Also immunosuppressive treatment, which often accompanies or replaces corticosteroidal therapy, does not always ensure phlogosis containment and control of symptoms, and further has the disadvantages of numerous contraindications and severe side effects (Podolsky, 2002).

The new drug generation that became available in the 1990's, are biological agents. The more in-depth knowledge of IBD natural history and of the main pathophysiological mechanisms has contributed to steer medical intervention in a concrete way. Thus, a development of 5 biotherapies aimed at controlling specific inflammatory "pathways" occurred through the use of recombinant human proteins, monoclonal chimeric humanized antibodies and fusion proteins. Contextually, agents which have showed a better efficacy in CD treatment are monoclonal chimeric antibodies directed to block TNF- $\alpha$ , a pro-inflammatory cytokine 10 overproduced during IBD (Seegers et al., 2002). This compound, which is at present in phase IV of clinical trial, is effective in the inflammation containment in about 60-70% of the treated patients. Nevertheless, some side effects have been pointed out with a considerable frequency of 15 incidence and recognizable in reactivation of latent microbial infections, hypersensitivity phenomena and formation of autoantibodies. The latter phenomenon could be based on the fact that anti-TNF- $\alpha$  neutralizes the cytokine TNF- $\alpha$  which has numerous biological functions.

In addition to its inflammatory effect, TNF- $\alpha$  takes part also to those mechanisms involved in the induction and maintaining 20 immunological tolerance. Therefore a block of TNF- $\alpha$  activity could paradoxically encourage excessive immunological reactions (Sandborn et al., 2002).

All these remarks suggest the need of new studies on animal 25 models of IBD through which it is possible to identify new active principles to be used in a better and durable treatment of such pathologies (Fiocchi, 2001).

Anti-TNF- $\alpha$  treatment, as far as the other biotherapies, such as the administration of anti-inflammatory cytokines, for example IL-10, represents a therapeutic extracellular approach aimed at controlling 30 biological effects of molecules secreted by inflammatory cells.

The study of the signal-transduction pathways activated by cytokine interaction with their receptors has outlined the chance to use new therapeutic strategies capable to modulate specifically and selectively 35 the intracellular expression of important inflammatory and non inflammatory molecules.

Under normal conditions, the intestinal mucosa is the seat of a "physiological" inflammatory infiltrate, which is maintained by a fragile balance between pro-inflammatory and anti-inflammatory molecules.

5 In relation to the above, an important role is played by TGF- $\beta$ 1, a multifunctional cytokine capable of regulating the growth, differentiation and activity of many immune and non immune cells.

Both *in vitro* and *in vivo* studies have demonstrated that TGF- $\beta$ 1 acts as a potent immunoregulator able to control mucosal intestinal inflammation, and that the inhibition of its activity results in the 10 development of colitis which shows immunomorphological similarity with CD or UC (Powrie F. et al., 1996; Neurath M. F. et al., 1996; Ludviksson B. R. et al., 1997).

In fact, TGF- $\beta$ 1 genes deficient mice display severe multifocal inflammatory responses, also involving the intestine, associated with an 15 excessive inflammatory cytokines production by numerous cell types, including T cells (Shull M. M. et al., 1992; Christ M. et al. 1994).

Similarly, inhibition of TGF- $\beta$ 1 signalling in mouse by expressing a dominant negative mutant form of the TGF- $\beta$ 1 receptor RII, results in an enhanced susceptibility to develop experimental colitis (Hahm 20 K.B. et al., 2001).

Finally, it was shown that specific inhibition of TGF- $\beta$ 1 signaling in T cells by the expression of a dominant negative TGF- $\beta$  receptor type II causes an autoimmune disease characterised by severe inflammatory infiltrations in lung and colon and the presence of circulating autoimmune 25 antibodies (Gorelik L. et al., 2000). These data indicate that the loss of activity of a single anti-inflammatory molecule could be sufficient to alter intestinal homeostasis and to allow immune responses leading to tissutal damage.

TGF- $\beta$ 1 anti-inflammatory activity starts with the interaction of 30 the molecule with a complex of heterodimeric transmembrane serine/threonine kinases receptors consisting of two subunits, named TGF- $\beta$ 1 R1 and TGF- $\beta$ 1 R2 respectively. Upon TGF- $\beta$ 1 binding, the receptors rotate relatively within the above mentioned complex, resulting in a trans-phosphorylation process and subsequent activation of TGF- $\beta$ 1 35 R1 by the constitutively active TGF- $\beta$ 1 R2 and capable of autophosphorylation.

5       The propagation of the TGF- $\beta$ 1-triggered signal to the nucleus is mediated by proteins belonging to the Smad family. Activated TGF- $\beta$ 1 R1 directly phosphorylates Smad2 and Smad3 proteins, which become able to interact with Smad4, thus enabling the complex Smad2-3/Smad4 to translocate to the nucleus, where it participates to the transcriptional control of some genes (Heldin C-H. et al., 1997).

10      The role of Smad3 in the TGF- $\beta$ 1 anti-inflammatory activity was supported by studies in animal models, which show that the deletion of the encoding gene for Smad3 is associated with diminished cell responsiveness to TGF- $\beta$ 1, and with a related development of inflammatory disease characterized by a massive infiltration of T-cells and pyogenic abscesses formation at gastrointestinal level (Yang X. et al., 1999).

15      Also other intracellular proteins, for example Smad7, belong to the Smads protein family. Such protein occupying TGF- $\beta$ 1 R1 interferes with the binding of Smad2/Smad3 to the receptor, thus preventing the phosphorylation and the activation. Hence, an increased expression of Smad7 protein is associated with an inhibition of the TGF- $\beta$ 1-mediated signaling (Hayashi H. et al., 1997).

20      The evaluation of the TGF- $\beta$ 1 expression in intestinal mucosa from IBD patients shows that said molecule production is paradoxically enhanced in comparison to what can be proved in the gut of normal patients (Lawrance IC. et al., 2001).

25      In a recent article the author of the present invention shows that mucosal samples from IBD patients are characterized by high levels of Smad7 and by reduced levels of active Smad3, thus indicating that during IBD the mechanism of TGF- $\beta$ 1-mediated signaling is compromised. The author of the present invention further showed that selective Smad7 abrogation by a specific antisense oligonucleotide 5'-  
30     GTCGCCCTTCTCCCCGCAGC-3' (SEQ ID No 1) restores lamina propria mononuclear cells (LPMC) responsiveness to TGF- $\beta$ 1, resulting in a down-regulation of pro-inflammatory cytokine production, such as for example, TNF- $\alpha$ .

35      Moreover, also ex vivo experiments carried out on intestinal mucosa samples from IBD patients showed that administration of Smad7 antisense ODN restores TGF- $\beta$ 1 signaling mechanism and allows a diminished cytokine production (Monteleone et al., 2001).

During IBD, intestinal mucosa is infiltrated with an high number of T cells. These cells are regarded to be the main mediators of tissutal damage acting in such diseases.

5 The increased number of T cells in the intestinal mucosa from IBD patients is partly dependent on the resistance of such cells against stimuli inducing their death (apoptosis).

10 It is believed that the block of T cells apoptosis plays a key role in maintaining the mucosal inflammatory response in IBD (Boirivant et al., 1999). Indeed, enhancing T cell death associates with a resolution of the intestinal inflammation. The exact mechanism underlying the resistance of T cells against apoptosis during IBD is not yet known, even if locally released cytokines seem to be involved.

15 Data from cell-culture *in vitro* experiments and *in vivo* studies indicate that TGF- $\beta$ 1 can either prevent or trigger T cell death and that the capacity of the factor to mediate both responses is site-specific (Han SH. et al., 1998; Arsura M. et al., 1996).

20 Smad3 knockout mice exhibit a massive increase in the number of inflammatory cells at the intestinal level, thus suggesting a role for TGF- $\beta$ 1 in controlling intestinal T cell apoptosis at intestinal level (Yang et al., 1999).

25 Therefore Smad7 inhibition by the use of Smad7 synthetic antisense ODN may represent a novel and acceptable "endogenous" biotherapeutic approach to chronic inflammatory diseases, in particular to IBD, since, as above mentioned, it restores T cells responsiveness to TGF- $\beta$ 1.

30 Antisense oligonucleotides (ODN) are short synthetic oligonucleotidic sequences complementary to the messenger RNA (m-RNA) which encodes for the target protein of the specific and aimed inhibition. Such sequences, hybridizing to the m-RNA, make a double-strand hybrid trait, and this lead to the activation of ubiquitary catalytic enzymes, such as RNases H, which degrade DNA/RNA hybrid strands that develop in nature to trigger DNA duplication, thus preventing protein translation.

35 The selection of the most suitable m-RNA regions and sequences to hybridize to the ODN has empirical characteristics even if ODN complementary to the transcriptional initiation region 5' and to the splicing regions usually result more effective. The design of a remarkable

number of antisense ODN, after identifying possible target sites, does not raise difficulties, thanks to the recent and advanced automated synthesis technologies owned by specialized companies in such field.

5 On the contrary the identification of the more active ODN, for possible therapeutic applications, requires a long-term screening work through efficacy assays in quantitative test. In relation to the above, antisense ODN sequences against specific target, among which Smad7, are already known (USA Patent No. US 6,159,697; assignee ISIS Pharmaceuticals Inc.).

10 The use of antisense ODN both for *in vitro* and *in vivo* gene regulation is thwarted by some problems, such as, the difficulty to pass through cellular membranes, due to the polianionic and then hydrophilic nature of these molecules, and the rapid enzymatic degradation.

15 To overcome these obstacles it is necessary to resort to chemical modification of the antisense ODN, such as, for example, phosphorothioation, as in the case of the above mentioned Smad7 specific sequence (Monteleone et al., 2001), or phosphoroamidation, that are substitutions of sulphur or nitrogen atoms in place of those oxygen atoms which are not the bridge atoms of the phosphodiester linkage.

20 As well as many biotechnological products, the demonstration of a biological activity points out a potential therapeutic activity.

Indeed ODN can be used either in the studies of both gene and protein functions involved in the pathogenesis of different diseases or for therapeutic purpose. Whereas in the former application field the antisense methodology was successful for the easiness of the guide principles, the shift from *in vitro* to *in vivo* experimentation is more complex, especially as regards pharmacokinetic, pharmacodynamic and toxicological aspects of these new drugs (Maggi A., 1998).

30 For example the Smad7 antisense ODN used in the previous experiments carried out by the author of the present invention (SEQ ID No 1), which shows *in vitro* biological activity, could show an increased risk of undesirable effects *in vivo*. In fact, such ODN contains two nucleotidic CG pairs which become CpG after phosphorothioation, an essential process to enhance ODN stability. The latter are sequences endowed with a powerful stimulating activity of the immune system, therefore the use of the above mentioned ODN as such could made worse the course of any immunologic disease, Crohn's disease and ulcerative colitis included.

5 A similar therapeutic approach could not be hypothesized, especially in the case of Crohn's disease, a pathology mediated by a particular class of T lymphocytes, named Th1, under the interleukin 12 stimulus. Indeed CpG molecules, as powerful inductors of the IL-12 synthesis, could induce a further development of Th1 cells.

10 In addition, *in vivo* administration of the antisense ODN containing CpG dinucleotides is accompanied by an increased risk of side effects, in comparison to oligonucleotides without CpG. In particular, it has been proved an increased risk of hyperplasia of the reticuloendothelial system of the spleen, kidney and liver, as well as an increased proliferation of hematopoietic cells (Agrawal S. et al., 2002).

15 Another problem in the use of ODN is bound to the side effects resulting from the action of the metabolites derived from the degradation of the molecule, which results quite susceptible to nuclease attack, since it is not protected at the 5' and 3' ends.

20 Therein the necessity of chemical modification of the phosphorothioate antisense ODN backbone to CpG pairs and to 5' and 3' ends. Nevertheless the above said modifications of the ODN sequence could lead up to the reduction or the loss of the biological activity of inhibition of Smad7 synthesis and, sometimes, even to the inversion of the desired activity both *in vitro* and *in vivo*.

25 Likewise it may be important to dispose of experimental IBD models suitable for *in vivo* studies, which allow to enlarge the knowledge on the mechanisms involving the loss of the regulation of the immunitary response and their role in the onset of IBD pathology and on the possibility to modulate or prevent such response, thus limiting inflammation progression at mucosal level. In relation to the above the TNBS-mediated colitis represents a spread and valid model of mucosal inflammation which shows striking immunomorphological similarities with 30 human CD (Neurath M. et al., 2000).

35 In the light of the above, it would be desirable to dispose of new therapeutic biological agents, like Smad7 antisense ODN, which are active both *in vitro* and *in vivo*, for the treatment of IBD through an "endogenous" biotherapeutic approach".

The author of the present invention has now found suitably modified antisense ODN sequences which exhibit an higher *in vivo* biological activity of inhibition of Smad7 expression in experimental

models of IBD in comparison to their *in vitro* inhibitory activity, and also higher than that of other known sequences showing the same modifications and tested on the same models.

5 In particular, the ODN sequences which exhibit an higher *in vivo* biological activity were designed according to the phosphorothioate antisense ODN sequence SEQ ID No 1 targeting the site 403 of the human Smad7 RNA, used by the author of the present invention in the course of previous experiments.

10 In view of the potential and future use of such Smad7 phosphorothioate antisense ODN for the treatment of human pathologies said sequence was modified at CpG dinucleotides therein contained, hereinafter indicated as XY, because of their already mentioned immunogenicity.

15 The study carried out by the author has allowed to test *in vivo* and *in vitro* efficacy of different known and novel Smad7 antisense ODN and their possible toxicity; and to investigate if blocking Smad7 expression results in a resolution of mucosal inflammation in experimental models of IBD.

20 The above mentioned suitably modified antisense ODN sequences according to the present invention, in addition to an higher biological activity *in vivo*, showed a surprisingly absence of side effects in animals, in spite of what happens after the administration of other sequences during the course of the same study. Further, ODN sequences according to the invention showed their efficacy to limit lymphocytic infiltration and the ulterior inflammation propagation, that is an evidence 25 not found for the other antisense ODN sequences herein tested.

The role of Smad7 as biological target clearly appears from these studies in experimental models, together with the possible therapeutic effects of its inhibition.

30 Furthermore, within the context of the present invention, another role of Smad7 on the induction of T cell apoptosis during IBD has been found. In fact, through the use of some Smad7 antisense ODN, it has been showed that TGF- $\beta$ 1 regulates intestinal T cell apoptosis and that a defective factor activity accounts for cell-resistance to apoptotic 35 stimuli, which are responsible for maintaining the mucosal inflammatory response.

Therefore the objects of the present invention are Smad7 phosphorothioate antisense oligonucleotides up to 21 nucleotides in length which comprise a portion of at least 10 nucleotides of the following sequence (SEQ ID No 2):

5 5'-GTXYCCCTTCTCCCXYCAG-3'

wherein X is a nucleotide comprising a nitrogenous base selected from the group consisting of cytosine, 5-methylcytosine and 2'-O-methylcytosine and wherein Y is a nucleotide comprising a nitrogenous base selected from the group consisting of guanine, 5-methylguanine e 2'-O-methylguanine, provided that at least one of the nucleotides X or Y comprises a methylated nitrogenous base;  
10 or the complementary sequence thereto.

Other objects of the present invention are the oligonucleotidic sequences of the different antisense oligonucleotide stereoisomers, such 15 as diastereoisomers and enantiomers, as to the phosphor atoms of the internucleosidic linkage included in the sequence. Indeed the internucleosidic linkage can be phosphorothioate or methylphosphonate and in both cases the phosphor bound to four different chemical groups represents a chiral centre.

20 Antisense oligonucleotides according to the present invention can have at least one methylphosphonate nucleotide into the sequence, which is placed, for example, either at only one of the 5' or 3' ends or at both 5' and 3' ends or along the oligonucleotidic sequence.

In a preferred embodiment the methylphosphonate nucleotide 25 can be either X or Y, in such a way that internucleosidic linkage is the linkage between said nucleotides.

Further modifications can be carried out to the 5' and 3' ends 30 and/or along the sequence of the antisense ODN to increase the stability of the molecule thus preventing the degradation by nucleases and reducing the risk of undesirable effects derived from the metabolite actions.

Antisense oligonucleotides according to the present invention 35 can have further at least one nucleotide of the sequence which is a 2'-O-methylribonucleotide 5'-monophosphate, which is placed, for example, either at only one of the 5' or 3' ends or at both 5' and 3' ends or along the oligonucleotidic sequence.

10

Further objects of the present invention are the above said antisense oligonucleotide wherein 2'-deoxyribonucleotides are replaced by ribonucleotides and 2'-deoxythymidine is replaced by uridine in such a way that antisense deoxyribonucleotidic sequences turn to the 5 correspondent antisense ribonucleotidic sequences.

A preferred embodiment of the present invention is represented by antisense oligonucleotides having the sequence (SEQ ID No 3):

5'-GTXGCCCTTCTCCCCXGCAG-3'

wherein X is 5-methyl 2'-deoxycytidine 5'-monophosphate.

10 Another preferred embodiment is represented by antisense oligonucleotides having the sequence (SEQ ID No 4):

5'-ZTXGCCCTTCTCCCCXGCAZ-3'

wherein X is 5-methyl 2'-deoxycytidine 5'-monophosphate and Z is 2'-deoxyguanosine methylphosphonate.

15 According to another aspect, a preferred embodiment of the present invention is antisense oligonucleotide having the sequence (SEQ ID No 15):

5'-ZTXGCCCTTCTCCCCXGCAZC-3'

wherein X is 5-methyl 2'-deoxycytidine 5'-monophosphate and Z is 2'-deoxyguanosine methylphosphonate.

20 Antisense ODN sequences according to the present invention can be advantageously used in medical field; therefore further objects of the present invention are pharmaceutical compositions which comprise at least one of the above disclosed antisense oligonucleotides as active principle together with one or more pharmaceutically acceptable adjuvants and/or excipients, which are known to skilled person in this field.

25 Further the invention relates to the use of the aforesaid antisense oligonucleotide sequences for the preparation of a medicament for the treatment of the pathologies associated with Smad7 expression. In particular, such pathologies associated with Smad7 expression are IBD, such as, for example, CD and UC.

30 The present invention is now described, for illustrative but not limitative purposes, according to its preferred embodiments, with particular reference to the figures of the enclosed drawings, wherein:

35 Figure 1 shows the effect on the number of intestinal T lymphocytes after 40 hours of treatment of mucosal samples from CD

patients with Smad7 ODN antisense and sense 5'-MePGTMe-dGCCCTTCTCCMe-dCGCAMEPG-3' (SEQ ID No 4);

Figure 2 shows the analysis of the expression of p-Smad2/Smad3 complex and of the total Smad2/Smad3 complex in LPMC isolated from the intestine of TNBS-treated mice (TNBS), untreated (Unt), treated with ethanol (EtOH) as controls;

Figure 3 shows the analysis of the Smad7 expression in LPMC isolated from the intestine of TNBS-treated mice (TNBS), untreated (Unt), treated with ethanol (EtOH) as controls;

Figure 4 shows the percentage changes in weight of mice with TNBS-induced colitis treated or not with Smad7 antisense oligonucleotide MePGTMe-dGCCCTTCTCCMe-dCGCAMEPGC (SEQ. ID No 15) or with a control (sense); the figure is representative of three separate experiments wherein fourteen mice for each group have been studied;

Figure 5 shows macroscopic aspect of the colon extracted from a mouse with TNBS-induced colitis and from a mouse with TNBS-induced colitis treated with Smad7 antisense oligonucleotide (SEQ. ID No 15); the figure is representative of three separate experiments wherein fourteen mice for each group have been studied;

Figure 6 shows histological aspect of a colon section from mice without colitis or with TNBS-induced colitis treated or not with Smad7 antisense oligonucleotide (SEQ. ID No 15) or with a control (sense); the figure is representative of three separate experiments wherein fourteen mice for each group have been studied. Magnification 40X.

25

EXAMPLE 1: Study on the effect of Smad7 antisense oligonucleotides according to the present invention on intestinal T cells apoptosis

#### MATERIALS AND METHODS

30

##### Synthesis of antisense ODN

All the Smad7 antisense ODN were synthesized by MWG Biotech AG (MWG Biotech S.r.l., Florence) employing standard automated techniques with an automated DNA synthesizer using standard phosphoroamidite chemistry protocols (Lesiak K. et al., 1993; Xiao W. et al., 1996).

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-dC) were synthesized according to known synthesis methods (Sanghvi et

al., 1993) using commercially available phosphoroamidites, whereas synthesis of modified oligonucleotides containing methylphosphonate groups (MeP) was accomplished using known protocols (Maier MA. et al., 2002).

5 The purification of the oligonucleotidic molecules has been carried out HPSF technology, developed by MWG Biotech. Such purification method has revealed an high efficiency since it allows removing failure sequences synthesized during the automated chemical synthesis process, such as, for example, n-1, n-2, n-x sequences, that standard purification classic methods are not capable to remove.

10 The above mentioned technology, besides enabling to obtain 100% of the desired length sequences without undesirable failure products, allows avoiding next desalting operation, since the purified sequences are free of both salt and metal ions.

15 Given the absence of any salt, oligonucleotides were eventually analyzed by MALDI-TOF mass spectrometry techniques according to standard protocols (Guerlavais T. et al., 2002; Ragas JA. et al., 2000). Then oligonucleotides were sterilized and the resulting solution was quantified as optical density (OD) by UV/visible spectrophotometer. Finally 20 the molecules were resuspended in sterile PBS1x before using.

All the used antisense ODN target Smad7 m-RNA sites which have 100% homology between human and mouse. In all the following oligonucleotides the internucleoside linkage is a phosphorothioate linkage.

25 The antisense ODN sequences being used in the present study have been designed according to the phosphorothioate antisense ODN sequence 5'-GTCGCCCTTCTCCCCGCAGC-3' (SEQ ID No 1) targeting the site 403 of the human Smad7 m-RNA, used by the author of the present invention in the course of previous experiments (Monteleone et al., 2001).

30 The Smad7 antisense ODN sequence 5'-MePGTMe-dCGCCCTTCTCCCMe-dCGCAMePG-3' (SEQ ID No 4) targets the site 403 of the human Smad7 m-RNA. This is a mixed-backbone oligonucleotide wherein the cytosine belonging to CpG pairs of SEQ ID No 1 were replaced by 5-methylcytosine (herein indicated as Me-dC). In addition, methylphosphonate linkages were placed at the ends of the molecule (herein indicated as MeP).

The Smad7 antisense ODN sequence 5'-GTTTGGTCCTAACATGC-3' (SEQ ID No 5) targets the site 294 of the human Smad7 m-RNA.

Mucosal samples were taken from resection specimens of 6 patients with moderate-to-severe CD and 4 patients with severe UC. In addition, intestinal mucosal samples were taken from 10 unaffected IBD patients undergoing colectomy for colon carcinoma (ethical approval was obtained by local committee). LPMC were prepared using the DTT-EDTA-collagenase procedure and resuspended in RPMI 1640 (Sigma-Aldrich S.r.l., Milan) supplemented with a serum replacement reagent HL-1 (Biowhittaker, Wokingham, UK).

Cells were cultured in the presence and absence of TGF- $\beta$ 1 (Sigma-Aldrich, final concentration ranging from 1 to 5 ng/ml) and after 48 hours of incubation were analyzed for the level of apoptosis.

In other experiments, LPMC isolated from IBD patients intestine were resuspended in RPMI 1640 supplemented with HL-1 and cultured in the presence and absence of the above mentioned Smad7 antisense ODN (SEQ ID No 4, SEQ ID No 5), and in the presence of a control sense oligonucleotide (both used at a concentration of 2  $\mu$ g/ml). After 24 hours, an aliquot of LPMC was used for extracting proteins and evaluate Smad7 expression. The remaining cells were extensively washed and resuspended in RPMI 1640 plus HL-1 and cultured in the presence or absence of TGF- $\beta$ 1 (5 ng/ml) for 48 hours and then analyzed for apoptosis.

25 Analysis of apoptosis by flow cytometry

Apoptosis was analyzed by propidium iodide (PI) staining followed by flow cytometry.

Cells were washed, incubated for 15 minutes at 37 °C in 5  $\mu$ l ribonuclease A (0,6  $\mu$ g/ml, 30-60 Kunitz units, Sigma-Aldrich), and then chilled on ice. Propidium iodide (100  $\mu$ g/ml) was added before analysis by flow cytometry.

T cells were identified using a specific monoclonal anti-CD3 antibody (DAKO Ltd., Cambridgeshire, UK).

35 Protein extraction and Western Blot analysis

LPMC were homogenized and total proteins were extracted in buffer A containing 10 mM Hepes (pH 7,9), 10 mM KCl, 0,1 mM EDTA and

0,2 mM EGTA. Buffer was supplemented with 1 mM dithiothreitol (DTT), 10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM phenylmethanesulphonyl fluoride (all reagents from Sigma-Aldrich).

Smad7 protein was analyzed using a specific rabbit anti-human Smad7 antibody (1:400 final dilution, Santa Cruz Biotechnology, Inc., CA; USA). Goat anti-rabbit antibodies conjugated to horseradish peroxidase (Dako Ltd) were used at 1:20.000 final dilution to detect primary antibody binding and immunoreactivity was visualized with a chemiluminescence kit (Pierce, Rockford, IL, USA).

**10      Organ culture**

Mucosal explants taken from the surgical specimens of patients were cultured in the presence or absence of Smad7 antisense ODN (SEQ ID No 4, SEQ ID No 5; both used at a final concentration of 10 µg/ml) for 40 hours.

**15**      As negative control, a mucosal explant was cultured in the presence of Smad7 sense ODN.

At the end of the culture, mucosal explants were collected and used for analyzing the number of lamina propria T lymphocytes by immunohistochemistry.

**20**      For this purpose, mucosal sections were prepared and stained with a monoclonal anti-CD3 antibody (DAKO). Goat anti-mouse antibodies conjugated to alkaline phosphatase (DAKO) were used to detect primary antibody binding.

**RESULTS**

**25**      The results obtained in the different experiments show how TGF-β1 enhanced, dose-dependently, apoptosis of T lymphocytes isolated from the intestine of normal subjects.

**30**      Table 1 shows the percentage of apoptotic T lymphocytes after 48 hours of culture. Numbers are the results of 4 separate experiments in which T cells isolated from the intestine of four normal subjects were used.

**Table 1**

	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Unstimulated	18%	17%	19%	23%
TGF- β1 (0,2 ng/ml)	22%	24%	23%	25%

TGF- $\beta$ 1 (1 ng/ml)	31%	33%	28%	31%
TGF- $\beta$ 1 (5 ng/ml)	33%	34%	32%	37%

5 In contrast, T lymphocytes isolated from four IBD patients showed a partial resistance to the TGF- $\beta$ 1-induced apoptosis signal as shown in the results reproduced in Table 2 which shows the percentage of apoptotic T lymphocytes after 48 hours of culture.

Table 2

	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Unstimulated	11%	10%	9%	7%
TGF- $\beta$ 1 (0,2 ng/ml)	12%	9%	8%	5%
TGF- $\beta$ 1 (1 ng/ml)	10%	11%	11%	8%
TGF- $\beta$ 1 (5 ng/ml)	16%	13%	14%	15%

10

In particular, from the analysis of data shown in Table 2 no meaningful increase in apoptosis was seen when T cells from IBD patients were cultured in the presence of either 0,2 ng/ml or 1 ng/ml TGF- $\beta$ 1 concentration. In contrast, stimulation of T cells from IBD patients with 5 ng/ml TGF- $\beta$ 1 resulted in a small increase in apoptosis.

15 Treatment of T lymphocytes isolated from IBD patients with the Smad7 antisense-ODN-SEQ-ID No-4-restored the cell responsiveness to TGF- $\beta$ 1, resulting in enhanced cell apoptosis, as shown in percentage values of T lymphocytes reproduced in Table 3. Data refer to four separate experiments in which T cells isolated from the intestine of four IBD patients, were cultured with medium alone (unstimulated) or pre-treated with medium and sense (control) or antisense oligonucleotides overnight 20 and then stimulated with TGF- $\beta$ 1 (1 ng/ml).

Table 3

	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Unstimulated	10%	9%	8%	7%
Medium	11%	9%	8%	5%
ODN Sense	12%	10%	10%	8%
ODN Antisense	33%	32%	23%	19%

Furthermore, using *ex vivo* organ culture, the author of the  
 5 present invention demonstrated that treatment of IBD biopsies with Smad7 antisense ODN according to the present invention significantly decreased the number of mucosal CD3+ T cells, as shown in the immunohistochemistry of Figure 1. The latter shows that the treatment with the antisense ODN reduces the number of mucosal CD3+ T cells.

10 Together these observations suggest the possibility that high Smad7 level plays a crucial role in prolonging T cell survival, thereby contributing to the propagation of local inflammation in IBD.

Thus, blocking Smad7 could represent a promising strategy to control mucosal inflammation in these condition.

15 **EXAMPLE 2: In vivo and in vitro studies on the effects of the administration of Smad7 antisense and sense oligonucleotides in experimental models of TNBS-induced colitis.**

#### MATERIAL AND METHOD

All the Smad7 antisense and sense ODN were synthesized by  
 20 MWG Biotech S.r.l. (Firenze) employing the standard techniques previously described.

The used antisense ODN target Smad7 m-RNA sites which have 100% homology between human and mouse. In all the following oligonucleotides the internucleoside linkage is a phosphorothioate linkage.  
 25 All the following sequences were used in the experiments carried out on the experimental induced-colitis models.

The Smad7 antisense ODN SEQ ID No 1 (5'-GTCGCCCTTCTCCCCGCAGC-3') targets the site 403 of the human

Smad7 m-RNA already used by the author of the present invention in the course of experiments published in a previous article (Monteleone et al., 2001).

For the further study concerning the role of Smad7 on the regulation of T cell apoptosis in LPMC isolated from the intestine of IBD patients the following antisense oligonucleotide sequences SEQ ID No 4 e 5 SEQ ID No 5 were used.

The Smad7 antisense ODN sequence 5'-MePGTMedCGCCCCCTCTCCCMed-dCGCAGMePG-3' (SEQ ID No 4) targets the site 403 of the human Smad7 m-RNA. This is a mixed-backbone oligonucleotide wherein the cytosine belonging to CpG pairs in the position 3 and 16 of SEQ ID No 1 were replaced by 5-methylcytosine (indicated as Me-dC). In addition, methylphosphonate linkages were placed at the ends of the molecule (indicated as MeP).

15 The Smad7 antisense ODN sequence .. 5'-  
GTTTGGTCCTGAACATGC-3' (SEQ ID No 5) targets the site 294 of the human Smad7 m-RNA. The internucleoside linkages included therein are phosphorothioate linkages.

20 The Smad7 antisense ODN sequence 5'-  
GTTTGGTCCTGAACAT-3' (SEQ ID No 6) targets the site 296 of the human Smad7 m-RNA.

The Smad7 antisense ODN sequence 5'-  
GTTTGGTCCTGAACATG-3' (SEQ ID No 7) targets the site 295 of the human Smad7 m-RNA.

25 The Smad7 antisense ODN sequence 5'-  
AGCACCGAGTGCGTGAGC-3' (SEQ ID No 8) targets the site 577 of the human Smad7 m-RNA.

The Smad7 antisense ODN sequence 5'-  
MePAGCACMedCGAGTGMedCGTGAGCMeP-3' (SEQ ID No 9) targets 30 the site 577 of the human Smad7 m-RNA. This is a mixed-backbone oligonucleotide wherein the cytosine in the position 6 and 12 of SEQ ID No 8 were replaced by 5-methylcytosine. In addition, methylphosphonate linkages were placed at the ends of the molecule.

35 The Smad7 antisense ODN sequence 5'-  
CGAACATGACCTCCGCAC (SEQ ID No 10) targets the site 233 of the human Smad7 m-RNA.

The Smad7 antisense ODN sequence 5'-Me-d CGA ACA TGA CCT CMe-d CG CAC-3' (SEQ ID No 11) targets the site 233 of the human Smad7 m-RNA. This is a mixed-backbone oligonucleotide wherein the cytosine in the position 1 and 14 of SEQ ID No 10 were replaced by 5-methylcytosine.

5 The Smad7 antisense ODN sequence 5'-GTMe-dCGCCCCTTCTCCMe-dCGCAG-3' (SEQ ID No 12) targets the site 403 of the human Smad7 m-RNA. This is a mixed-backbone oligonucleotide wherein the cytosine belonging to CpG pairs in the position 3 and 16 of 10 SEQ ID No 1 were replaced by 5-methylcytosine (indicated as Me-dC).

The Smad7 antisense ODN sequence 5'-GATCGTTGGTCCTGAA-3' (SEQ ID No 13) targets the site 299 of the human Smad7 m-RNA.

15 The Smad7 antisense ODN sequence 5'-ATCGTTGGTCCTGAAC-3' (SEQ ID No 14) targets the site 298 of the human Smad7 m-RNA.

20 The Smad7 antisense ODN sequence MePGTMe-dCGCCCCTTCTCCMe-dCGCAMEPGC (SEQ ID No 15) targets the site 403 of the human Smad7 m-RNA. This is a mixed-backbone oligonucleotide wherein the cytosine belonging to CpG pairs in the position 3 and 16 of SEQ ID No 1 were replaced by 5-methylcytosine (indicated as Me-dC). In addition, methylphosphonate linkages were placed at one of the ends of the oligonucleotides and at the guanine residue in position 20.

#### Induction of colitis

25 Five to six-week old male SJL/J mice were maintained in a specific pathogen-free animal facility. For induction of colitis, 2,5 mg TNBS (pH 1,5–2,0; Sigma Aldrich) in 50% ethanol was administered per rectum to lightly anesthetized mice through a 3,5 F catheter. The catheter tip was inserted 4 cm proximal to the anal verge, then 100 ml of fluid (TNBS/ethanol) was slowly instilled into the colon.

To ensure distribution of the TNBS within the entire colon and cecum, mice were held in a vertical position for 30 seconds after the injection. Some of the mice were administered with 50% ethanol alone using the same technique and were used as controls.

#### Histologic assessment of colitis

35 Tissues removed from mice at indicated times of death were fixed in 10% formalin solution (Sigma Aldrich), embedded in paraffin, cut

into tissue sections and stained with hematoxyline and eosine. Stained sections were examined for evidence of colitis using different criteria such as the presence of lymphocyte infiltration, elongation and/or distortion of crypts, frank ulceration and thickening of the bowel wall.

5 The degree of inflammation on microscopic cross-sections of the colon was graded from 0 to 4 as follows:

0: no evidence of inflammation;

10 1: low level of lymphocyte infiltration with infiltration seen in a <10% high-power field (hpf= high power field), no structural changes observed;

15 2: moderate lymphocyte infiltration with infiltration seen in <10–25% hpf, crypt elongation, bowel wall thickening which does not extend beyond mucosal layer;

15 3: high level of lymphocyte infiltration with infiltration seen in <25–50% hpf, thickening of bowel wall which extends beyond mucosal layer;

20 4: marked degree of lymphocyte infiltration with infiltration seen in >50% hpf, high vascular density, crypt elongation with distortion, transmural bowel wall-thickening with ulceration.

20

Isolation of lamina propria mononuclear cells (LPMC) and treatment of cells with Smad7 antisense ODN

25 The lamina propria mononuclear cells (LPMC) were isolated from colonic specimens. The specimens were first washed in HBSS-calcium magnesium free (Hanks' balanced salt solution, Sigma-Aldrich) and cut into 0,5-cm pieces. They were then incubated twice, each time for 15 minutes in HBSS containing EDTA (0,37 mg/ml) and dithiothreitol (0,145 mg/ml) at 37°C. The tissues were then digested in RPMI containing collagenase D (400 U/ml, Boehringer Mannheim Biochemicals, 30 Indianapolis, IN) and DNase I (0,01 mg/ml, Boehringer Mannheim Biochemicals, Indianapolis, IN) in a shaking incubator at 37°C.

35 The LPMC released from the tissue were resuspended in 100% Percoll, layered under a 40% Percoll gradient (Pharmacia Biotech AB, Uppsala, Sweden), and spun at 1,800 rpm for 30 minutes to obtain the lymphocyte-enriched population.

To assess the *in vitro* efficacy of Smad7 antisense ODN, the LPMC isolated from TNBS-treated mice, were resuspended in RPMI 1640

20

(Sigma-Aldrich) supplemented with a serum replacement reagent HL-1 (Biowhittaker) at a final concentration of  $1 \times 10^6$ /ml in 24 well plates. For transfection of antisense ODN, 2  $\mu$ l of lipofectamine 2000 reagent (LF, Invitrogen Italia SRL, San Giuliano Milanese) was used for each ml of cell medium following the protocol. Then, 2  $\mu$ g/ml of antisense ODN and LF were combined and allowed to incubate for 20 minutes at room temperature. The obtained mixture was then added directly to the cells. After overnight culture, the cells were removed from the plate and used for analysis of Smad7 by Western blotting.

10

Treatment of mice with Smad7 antisense ODN

Two days after treatment with TNBS, mice were administered per rectum 150  $\mu$ g of each Smad7 antisense or sense oligonucleotide. At least 5 mice for group were examined. At fifth day mice were sacrificed and whole intestinal mucosal samples were taken and analysed for Smad7 and Smad3 content by Western blotting. In addition intestinal mucosal inflammation degree entity was evaluated.

15

Protein extraction and Western Blot analysis

Both lamina propria mononuclear cells and whole colonic specimens were homogenized using the above procedure. Then Smad7 expression was revealed by Western blotting.

20

At the end, the blots were stripped using a commercially available solution (Pierce) and probed with anti-actin antibodies (Sigma-Aldrich) to verify the same amount of protein were filled in each well. Detection was accomplished using a chemiluminescence kit (Pierce). The intensity of bands was analysed by a densitometer.

25

Both LPMC and whole colonic specimen samples proteins were also analyzed for the content of phosphorylated and total Smad3 protein by Western blotting using specific commercially available antibodies (Santa Cruz).

30

For the analysis of phosphorylated Smad3 a specific rabbit anti-human antibody capable to recognize phosphorylated Smad2/3 proteins as antigen (1:500 final dilution), and a goat anti-rabbit antibody conjugated to horseradish peroxidase (1:20.000 dilution) were used. Immunoreactivity was visualized with a chemiluminescence kit (Pierce).

35

After detection, blots were stripped using a commercially available solution (Pierce) and incubated with a specific goat anti-human Smad3 antibody (1:500 final dilution) followed by a rabbit anti-goat

antibody conjugated to horseradish peroxidase (1:20.000 dilution); then immunoreactivity was visualized with the above mentioned chemiluminescence kit (Pierce).

#### Test ELISA

5       The amount of active TGF- $\beta$ 1 was determined in the intestinal mucosal samples. To this aim total proteins were extracted from mucosal samples from mice with or without TNBS-induced colitis as above indicated. The levels of active TGF- $\beta$ 1 were analyzed using a commercially available ELISA kit (R&D Systems, Space Import-Export Srl, 10 Milano). Optical density was measured on a Dynatech MR 5000 ELISA reader at a wavelength of 490 nm. Data were expressed as pg/100  $\mu$ g of total proteins.

#### RESULTS

15      After receiving TNBS mice developed diarrhoea and weight loss by evidence of the induction of colitis. The colon was macroscopically enlarged and histological analysis of its mucosa showed moderate to severe inflammatory lesions.

20      To examine if induction of TNBS-colitis was associated with changes in the production of TGF- $\beta$ 1, colonic specimens were taken from mice with or without colitis and analyzed for the content of active TGF- $\beta$ 1 by ELISA.

25      As several cell types which have the potential to synthesize TGF- $\beta$ 1 are present at intestinal level, it was used for the evaluation the whole intestinal mucosa rather than LPMC alone.

30      In the absence of colitis low levels of active TGF- $\beta$ 1 were detected ( $85\pm12$  and  $94\pm26$  pg/ $\mu$ g of total protein in unstimulated and controls mice respectively). Significantly enhanced TGF- $\beta$ 1 levels were measured in mucosal samples from mice with TNBS-induced colitis ( $985\pm120$  pg/ $\mu$ g of total protein) ( $p<0.01$ ). Even though this result seems to suggest that during TNBS-induced colitis there could be an increasing TGF- $\beta$ 1 activity, the analysis of intracellular levels of active Smad3 in intestinal LPMC isolated from mice with colitis surprisingly exhibit a reduced Smad3 phosphorylation to be associated with an enhanced induction of Smad7 (Figure 2 and 3). In particular, figure 2 illustrates the presence of a band corresponding to the active (phosphorylated) Smad2/3 in LPMC isolated from the unaffected intestine but not from mice with TNBS-induced colitis. In the figure 3 it has been showed that the two

bands, the lower 47 Kda band corresponding to the free Smad7 and the upper 102 Kda band corresponding to the TGF- $\beta$ 1 R1-Smad7 complex, are present only in LPMC specimens isolated from the intestine of mice with TNBS-induced colitis. These data indicate that local inflammation stimulates the synthesis of TGF- $\beta$ 1 which is however not able to dampen the mucosal inflammation.

According to the present invention it has been evaluated if treating TNBS mice with Smad7 antisense ODN could restore the endogenous TGF- $\beta$ 1 function and limit the ongoing inflammation.

First, it has been tested the efficacy of the above mentioned Smad7 antisense ODN (SEQ ID No 1 and SEQ ID No 4-15) to decrease Smad7 expression both *in vitro* and *in vivo* experiments.

As regards *in vitro* experiments, the LPMC isolated from the intestine of mice with TNBS-induced colitis were transfected with each of the Smad7 antisense ODN and incubated overnight. Smad7 analysis was carried out by Western blotting.

As regards *in vivo* experiments TNBS-treated mice were administered with Smad7 antisense ODN and after 3 days animals were sacrificed, tissue specimens were taken and Smad7 analysis was carried out by Western blotting.

Table 4 summarizes the results of these experiments and shows the percentage inhibition obtained by each Smad7 antisense oligonucleotide both *in vitro* and *in vivo* experiments. Data indicate mean  $\pm$  standard deviation (SEM) of four separate *in vitro* experiments and mean  $\pm$  SEM of five separate *in vivo* experiments.

Table 4

Sequence (5' → 3')	Site	%inhibition <i>in LPMC</i>	%inhibition <i>in vivo</i>	SEQ ID No
gtcgccccccttccccgcagc	403	29 $\pm$ 3	33 $\pm$ 0.5	1
MePgtMedcgccccctctcc cMe-dcgcaMePg	403	34 $\pm$ 1.5	55 $\pm$ 3	4
gtt tgg tcc tga aca tgc	294	26 $\pm$ 2.6	25 $\pm$ 3.4	5
gtt tgg tcc tga acat	296	16 $\pm$ 2	15 $\pm$ 3.2	6

23

gtt tgg tcc tga acatg	295	17 ± 3.1	10 ± 1.12	7
agc acc gag tgc gtg agc	577 (*)	27 ± 0.88	25 ± 2.7	8
MePacgacMedc gag tgMedc gtg agcMeP	577 (*)	29 ± 1.65	30 ± 1.3	9
cga aca tga cct ccg cac	233 (**)	33 ± 2.3	32 ± 1.89	10
Me-d cga aca tga cct cMe-d cg cac	233 (**)	36 ± 1.5	34 ± 2.2	11
gtMedcgcccccttcMe dcgcag	403	32 ± 4.1	42 ± 1.8	12
gatcgtttgtcctgaa	299			13
atcgtttgtcctgaa	298			14
MePgtMedcgcccccttc cMedcgcaMePgc	403	34 ± 1.6	56 ± 3	15

(\*) Sequences No 16 and (\*\*) No 12 of the Patent US6159697

by ISIS.

All the antisense ODN were effective in reducing Smad7 expression when transfected *in vitro* in LPMC isolated from TNBS-treated murine models. From the analysis of the value of percentage inhibition shown in Table 4 it is remarkable that antisense oligonucleotidic sequences SEQ ID No 4, 10, 11, 12 and 15 showed the major efficacy.

Nevertheless, the percentage of Smad7 expression inhibition obtained by *in vivo* treatment with oligonucleotidic sequences SEQ ID No 10 and 11 did not significantly differ from that documented *in vitro* experiments.

Instead, treatment of mice with antisense ODN SEQ ID No 4 and 12 and 15 resulted clearly in a greater percentage of Smad7 inhibition than that obtained *in vitro* experiments, that is 55% vs 34%, 42% vs 32% e 56% vs 34% respectively (P<0.01).

In contrast, treatment of mice with antisense oligonucleotide SEQ ID No 7 caused a reduction in Smad7 expresion *in vivo* which was of lower entity than that resulting when the antisense oligonucleotide was transfected in LPMC *in vitro*, that is 10% vs 17%, P<0.01.

5 Overall, these results suggest that only specific modification into a Smad7 antisense ODN sequence are able to improve its pharmacokinetic, biochemical and biophysical profile.

10 No sign of acute toxicity was documented in mice receiving antisense oligonucleotides (SEQ ID No 1 and SEQ ID No 4-15). One out of 5, treated with TNBS, died after 3 days (20%). Similarly, 1/5 of mice receiving the Smad7 sense oligonucleotide died after 4 days.

15 No mortality was documented in mice group treated with Smad7 antisense ODN SEQ ID No 1 and SEQ ID No 4-15.

20 The use of antisense ODN sequences SEQ ID No 13 and SEQ ID No 14 is associated with a reasonable *in vitro* inhibition activity (11% and 9,5%, respectively). Nevertheless, the *in vivo* administration of such sequences was unexpectedly joined with a marked deterioration of the colitis, up to cause the death of all the mice after 72 hours of treatment.

25 Macroscopic analysis of the intestinal samples taken from these mice has revealed the presence of a severe colitis and this was associated to a substantial increase in the intestinal Smad7 expression.

As above said, it was tested the efficacy of Smad7 antisense ODN to limit the ongoing inflammation. For this purpose, mice after induction of colitis were administered with antisense oligonucleotides SEQ ID No 1, 4, 5 and 15 considering 5 animals for each group.

30 Following the treatment with Smad7 antisense ODN it has been revealed a reduction of the mucosal inflammation. This result was particularly evident in mice treated with antisense oligonucleotides 4 and 15. Indeed, the colitis severity of grade 3-4 in mice with colitis not receiving antisense reached grade 2 or 3 after administration of antisense oligonucleotide sequences 1 or 5 respectively, while in mice treated with oligonucleotidic sequences 4 or 15, inflammation has not exceeded grade 1.

35 To examine if Smad7 antisense oligonucleotides were effective also when administered orally, mice with TNBS-induced colitis were treated the day after the induction of colitis with Smad7 antisense oligonucleotide 4 or 15 or control (sense).

For this purpose oligonucleotides were risuspended in a bicarbonate solution. The final volume of the solution administered to each mouse was of 350 µl and contains doses of oligonucleotide equivalent to 250, 500 or 1000 µg. Such solution was administered *per os* through a catheter.

At fifth day mice were sacrificed and analysis of Smad7 expression and of inflammation degree were evaluated as indicated in the previous paragraphs. All the mice treated with antisense oligonucleotide, and not with the control sense oligonucleotide, showed a meaningful reduction of Smad7 expression ad an increased Smad3 phosphorylation, independently from the dose of the oligonucleotide being used.

Substantially, Smad7 inhibition was associated with a weight recovery as shown in Figure 4. The figure 4 exhibits a graph which shows the percentage change in weight of the mice with TNBS-induced colitis treated or not treated with Smad7 antisense oligonucleotide (SEQ. N. 15) or control (sense). Both oligonucleotides were administered *per os* at the dose of 250 µg through catheter two days after the induction of colitis. The weight loss documentable at the second day in each of the three groups indicates that the treatment with TNBS induced colitis. Further it was proved that starting from the fourth day mice treated with Smad7 antisense oligonucleotide, but not with the control, showed a body weight recovery. The apparent and slight recovery seen at the fifth day in mice with TNBS-induced colitis is due to the fact that the 21,4% of mice with colitis died at the fourth day and therefore they were not considered in the evaluation of the body weight at the fifth day.

Smad7 inhibition was correlated to a marked suppression of tissutal inflammation as shown in figures 5 and 6. Figure 5 exhibits the images of the colon extracted from a mouse with TNBS colitis and from a mouse with TNBS colitis treated with Smad7 antisense oligonucleotides (SEQ. ID No 15). The oligonucleotide was administered *per os* at the dose of 250 µg through a catheter, at the second day after the induction of colitis. It has been showed that the colon from the mouse with TNBS-colitis is highly inflammed, shortened and thickening. On the contrary, the mouse receiving Smad7 antisense shows a colon of normal length and thickness and no macroscopic signs of phlogosis. Figure 6 exhibits histological aspect of a colon section from a mouse without colitis or from mice with TNBS-colitis treated or not treated with Smad7 antisense oligonucleotide

(SEQ ID No 15) or the control (sense). Both oligonucleotides were administered *per os* at the dose of 250 µg through catheter the second day after the induction of colitis. It has been shown that in the mouse without colitis, glands appear rectilinear and uniform with a normal content of muciparous cells and inflammatory elements of lamina propria. On the contrary, in the colon of TNBS treated mice receiving or not the control oligonucleotide, there was a total destruction of the glandular structure, with a muciparous and a massive inflammatory cells infiltration in the lamina propria. In the colic section of the mouse treated with TNBS and receiving Smad7 antisense oligonucleotide the presence of a normal glandular structure and the absence of phlogosis were demonstrated.

Together these observations suggest that the use of antisense ODN, which show the higher efficacy of Smad7 inhibition accompanied by the absence of side effects, following the *in vivo* administration, can represent a promising therapeutic strategy in the control of mucosa inflammation during IBD, especially if such characteristics of efficacy and toxicity were compared with the results achieved with other antisense ODN sequences with the same efficacy in the Smad7 *in vitro* inhibition.

## BIBLIOGRAPHY

- Podolsky D.K., N. Engl. J. Med., 2002 Ago; Vol.347: No 6.
- Seegers D. et al. Aliment. Pharmacol. Ther., 2002; Vol.16: 53-58.
- Sandborn J., et al. Gastroenterology, 2002 Mag; Vol.122: No 6.
- 5 - Fiocchi C., J. Clin. Invest., 2001 Ago; Vol.108: 523-526.
- Powrie F., et al. J Exp Med 1996; 183: 2669-2674.
- Neurath M. F., et al. J Exp Med 1996; 183: 2605-2616.
- Ludviksson B. R., et al. J Immunol 1997; 159: 3622-3628.
- Shull M. M., et al. Nature 1992; 359: 693-699.
- 10 - Christ M., et al. J Immunol 1994; 153: 1936-1946.
- Hahm K.B., et al. Gut. 2001; 49:190-198.
- Gorelik L., et al. Immunity 2000; 12: 171-181.
- Heldin C-H., et al. Nature 1997; 390: 465-471.
- Yang X., et al. Embo J 1999; 18: 1280-1291.
- 15 - Hayashi H., et al. Cell 1997; 89: 1165-1173.
- Lawrence IC. et al. Inflamm Bowel Dis 2001;7:16-26.
- Monteleone G., et al. J. Clin. Invest., 2001 Giu; Vol.108:601-609.
- Boirivant M., et al. Gastroenterology 1999; 116: 557-565.
- Han SH., et al. J Pharmacol Exp Ther. 1998; 287:1105-12.
- 20 - Arsura M., et al. Immunity 1996; 5: 31-40.
- Brevetto statunitense US 6,159,697.
- Maggi A., Biotecnologie Farmacologiche, 1998; Cap.8: 125-131.
- Agrawal S., Molecular Medicine Today, 2002; Vol. 6: 72-81.
- Neurath M., Fuss I., Strober W., Int Rev Immunol., 2000; Vol.19: 51-
- 25 - 62.
- Lesiak K. et al., Bioconjugate Chem., 1993; Vol. 4: 467.
- Xiao W. et al. Antisense Nucleic Acid Drug Dev., 1996; Vol. 6: 247-258.
- Sanghvi et al, Nuclei Acids Research, 1993; Vol. 21: 3197-3203.
- 30 - Maier MA. et al. Org Lett., 2002; Vol. 2: 1819-1822.
- Guerlavais T., et al. Anal Bioanal Chem., 2002; Vol. 374: 57-63.
- Ragas J.A., et al. Analyst., 2000; Vol.125: 575-581.

## CLAIMS

1. Antisense oligonucleotides phosphorothioate against Smad7 up to 21 nucleotides in length which comprise a portion of at least 10 nucleotides of the following sequence (SEQ ID No 2):
  - 5 5'-GTXYCCCCTTCTCCCCXYCAG-3'  
wherein X is a nucleotide comprising a nitrogenous base selected from the group consisting of cytosine, 5-methylcytosine and 2'-O-methylcytosine and wherein Y is a nucleotide comprising a nitrogenous base selected from the group consisting of guanine, 5-methylguanine e 2'-O-methylguanine, provided that at least one of the nucleotides X or Y comprises a methylated nitrogenous base; or the complementary sequence thereto.
  - 10 2. Antisense oligonucleotides according to claim 1, wherein at least one nucleotide of the sequence is methylphosphonate.
  - 15 3. Antisense oligonucleotides according to claim 2, wherein said at least one methylphosphonate nucleotide is placed either at only one of the 3' or 5' ends or at both 3' and 5' ends or along the antisense oligonucleotidic sequence.
  - 20 4. Antisense oligonucleotides according to claim 2, wherein the methylphosphonate nucleotide is Y.
  5. Antisense oligonucleotides according to claim 2, wherein the methylphosphonate nucleotide is X.
  - 25 6. Antisense oligonucleotides according to claim 1, wherein at least one nucleotide of the sequence is a 2'-O-methylribonucleotide 5'-monophosphate.
  7. Antisense oligonucleotides according to claim 6, wherein said at least one 2'-O-methylribonucleotide 5'-monophosphate is placed at only one of the 3' or 5' ends or at both 3' and 5' ends or along the oligonucleotidic sequence.
  - 30 8. Antisense oligonucleotides according to anyone of the preceding claims wherein 2'-deoxyribonucleotides are replaced by the corresponding ribonucleotides.
  9. Antisense oligonucleotides according to anyone of the claims from 1 to 8 having sequence (SEQ ID No 4):
    - 35 5'-ZTXGCCCTTCTCCCCXGCAZ-3'  
wherein X is 5-methyl 2'-deoxycytidine 5'-monophosphate and Z is 2'-deoxyguanosine methylphosphonate.

10. Antisense oligonucleotides according to anyone of the claims from 1 to 8 having sequence (SEQ ID No 15):

5'-ZTXGCCCTTCTCCCXGCAZC-3'

wherein X is 5-methyl 2'-deoxycytidine 5'-monophosphate and Z is 2'-  
5 deoxyguanosine methylphosphonate.

11. Antisense oligonucleotides according to claim 1 having sequence (SEQ ID No 3):

5'-GTXGCCCTTCTCCCXGCAG-3'

wherein X is 5-methyl 2'-deoxycytidine 5'-monophosphate.

10 12. Antisense oligonucleotides as defined in claims from 1 to 11 for use in medical field.

13. Pharmaceutical composition which comprises at least one of the antisense oligonucleotides as defined in claims from 1 to 11 as active principle together with one or more pharmaceutically acceptable adjuvants and/or excipients.

14. Use of the antisense oligonucleotides as defined in claims from 1 to 11 for the preparation of a medicament for the treatment of the pathologies associated with Smad7 expression.

15. Use according to claim 14, wherein the pathologies associated  
20 with Smad7 expression are chronic inflammatory diseases.

16. Use according to claim 15, wherein the chronic inflammatory diseases are Crohn's disease and ulcerative colitis.

Abstract of the Disclosure

The invention relates to antisense oligonucleotidic sequences (ODN) against Smad7 suitably modified, and their uses in medical field as therapeutic biological agents, in particular in the treatment of chronic inflammatory bowel disease, such as Crohn's disease and ulcerative colitis.

Giuliani ODN ST25  
SEQUENCE LISTING  
620 Rec'd PCT/PTO 29 SEP 2005

<110> Giuliani S. p. A.  
<120> Antisense oligonucleotides (ODN) against Smad 7 and uses in medical field thereof  
<130> PCT 25572  
<140> RM2003A000149  
<141> 2003-04-02  
<150> RM2003A000149  
<151> 2003-04-02  
<160> 15  
<170> PatentIn version 3.2  
<210> 1  
<211> 21  
<212> DNA  
<213> Artificial  
<220>  
<223> Antisense phosphorothioate sequence targeting the site 403 of the human Smad7 m-RNA  
  
<220>  
<221> misc\_feature  
<222> (1)..(21)  
  
<400> 1  
gtcgcccctt ctcccccgcag c 21  
  
<210> 2  
<211> 20  
<212> DNA  
<213> Artificial  
<220>  
<223> Modified phosphorothioate antisense oligonucleotide targeting human Smad 7 m-RNA  
  
<220>  
<221> misc\_feature  
<222> (3)..(3)  
<223> "n": c  
  
<220>  
<221> modified\_base  
<222> (3)..(3)  
<223> "n": m5c, cm  
  
<220>  
<221> misc\_feature  
<222> (4)..(4)  
<223> "n": g  
  
<220>  
<221> modified\_base  
<222> (4)..(4)  
<223> "n": gm, 5'-methylguanine  
  
<220>  
<221> misc\_feature

## Giuliani ODN.ST25.txt

```

<222> (16)..(16)
<223> "n": c

<220>
<221> modified_base
<222> (16)..(16)
<223> "n": m5c, cm

<220>
<221> misc_feature
<222> (17)..(17)
<223> "n": g

<220>
<221> modified_base
<222> (17)..(17)
<223> "n": gm, 5'-methylguanine

<400> 2
gttnccctt ctcccnnncag                                20

<210> 3
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Modified phosphorothioate antisense oligonucleotide targeting
Smad 7 m-RNA

<220>
<221> misc_feature
<222> (3)..(3)
<223> "n": 5-methyl 2'-deoxycytidine 5'-monophosphate

<220>
<221> modified_base
<222> (3)..(3)
<223> m5c

<220>
<221> misc_feature
<222> (16)..(16)
<223> "n": 5-methyl 2'-deoxycytidine 5'-monophosphate

<220>
<221> modified_base
<222> (16)..(16)
<223> m5c

<400> 3
gtngccctt ctcccngcag                                20

<210> 4
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Modified antisense phosphorothioate oligonucleotide targeting the
site 403 of the human Smad 7 m-RNA

<220>
<221> misc_feature
<222> (1)..(1)

```

Giuliani ODN.ST25.txt

```

<223> "n": 2'-deoxyguanosine methylphosphonate

<220>
<221> modified_base
<222> (1)..(1)
<223> 2'-deoxyguanosine methylphosphonate

<220>
<221> misc_feature
<222> (3)..(3)
<223> "n": 5-methyl 2'deoxycytidine 5' monophosphate

<220>
<221> modified_base
<222> (3)..(3)
<223> m5c

<220>
<221> misc_feature
<222> (16)..(16)
<223> "n": 5-methyl 2'deoxycytidine 5' monophosphate

<220>
<221> modified_base
<222> (16)..(16)
<223> m5c

<220>
<221> misc_feature
<222> (20)..(20)
<223> "n": 2'-deoxyguanosine methylphosphonate

<220>
<221> modified_base
<222> (20)..(20)
<223> 2'-deoxyguanosine methylphosphonate

```

<400> 4  
ntngccctt ctcccngcan

20

```

<210> 5
<211> 18
<212> DNA
<213> Artificial

<220>
<223> Antisense phosphorothioate oligonucleotide targeting the site
294 of the human Smad 7 m-RNA

```

<220>
<221> misc\_feature
<222> (1)..(18)

<400> 5  
gtttggtcct gaacatgc

18

```

<210> 6
<211> 16
<212> DNA
<213> Artificial

```

<220>
<223> Antisense phosphorothioate oligonucleotide targeting the site
296 of the human Smad 7 m-RNA

## Giuliani ODN.ST25.txt

<220>  
<221> misc\_feature  
<222> (1)..(16)

<400> 6  
gtttggcct gaacat 16

<210> 7  
<211> 17  
<212> DNA  
<213> Artificial

<220>  
<223> Antisense phosphorothioate oligonucleotide targeting the site 295 of the human Smad 7 m-RNA

<220>  
<221> misc\_feature  
<222> (1)..(17)

<400> 7  
gtttggcct gaacatg 17

<210> 8  
<211> 18  
<212> DNA  
<213> Artificial

<220>  
<223> Antisense phosphorothioate oligonucleotide targeting the site 577 of the human Smad 7 m-RNA

<220>  
<221> misc\_feature  
<222> (1)..(18)

<400> 8  
agcacccgagt gcgtgagc 18

<210> 9  
<211> 18  
<212> DNA  
<213> Artificial

<220>  
<223> Modified antisense phosphorothioate oligonucleotide targeting the site 577 of the human Smad 7 m-RNA

<220>  
<221> misc\_feature  
<222> (1)..(1)  
<223> "n": 2'-deoxyguanosine methylphosphonate

<220>  
<221> modified\_base  
<222> (1)..(1)  
<223> 2'-deoxyguanosine methylphosphonate

<220>  
<221> misc\_feature  
<222> (6)..(6)  
<223> "n": 5-methyl 2'deoxycytidine 5' monophosphate

## Giuliani ODN.ST25.txt

<220>  
<221> modified\_base  
<222> (6)..(6)  
<223> m5c  
  
<220>  
<221> misc\_feature  
<222> (12)..(12)  
<223> "n": 5-methyl 2'deoxycytidine 5' monophosphate

<220>  
<221> modified\_base  
<222> (12)..(12)  
<223> m5c

<220>  
<221> misc\_feature  
<222> (18)..(18)  
<223> "n": 2'-deoxyguanosine methylphosphonate

<220>  
<221> modified\_base  
<222> (18)..(18)  
<223> 2'-deoxyguanosine methylphosphonate

<400> 9  
ngcacngagt gngtggagn

18

<210> 10  
<211> 18  
<212> DNA  
<213> Artificial

<220>  
<223> Antisense phosphorothioate oligonucleotide targeting the site  
233 of the human Smad 7 m-RNA

<220>  
<221> misc\_feature  
<222> (1)..(18)

<400> 10  
cgaacatgac ctccgcac

18

<210> 11  
<211> 18  
<212> DNA  
<213> Artificial

<220>  
<223> Modified antisense phosphorothioate oligonucleotide targeting  
the site 233 of the human Smad 7 m-RNA

<220>  
<221> misc\_feature  
<222> (1)..(1)  
<223> "n": 5-methyl 2'deoxycytidine 5' monophosphate

<220>  
<221> modified\_base  
<222> (1)..(1)  
<223> m5c

<220>  
<221> misc\_feature

## Giuliani ODN.ST25.txt

<222> (14)..(14)  
<223> "n": 5-methyl 2'deoxycytidine 5' monophosphate

<220>  
<221> modified\_base  
<222> (14)..(14)  
<223> m5c

<400> 11  
ngaacatgac ctcngcac

18

<210> 12  
<211> 20  
<212> DNA  
<213> Artificial

<220>  
<223> Modified antisense phosphorothioate oligonucleotide targeting the site 403 of the human Smad 7 m-RNA

<220>  
<221> misc\_feature  
<222> (3)..(3)  
<223> "n": 5-methyl 2'deoxycytidine 5' monophosphate

<220>  
<221> modified\_base  
<222> (3)..(3)  
<223> m5c

<220>  
<221> misc\_feature  
<222> (16)..(16)  
<223> "n": 5-methyl 2'deoxycytidine 5' monophosphate

<220>  
<221> modified\_base  
<222> (16)..(16)  
<223> m5c

<400> 12  
gtngccctt ctcccngcag

20

<210> 13  
<211> 17  
<212> DNA  
<213> Artificial

<220>  
<223> Antisense phosphorothioate oligonucleotide targeting the site 299 of the human Smad 7 m-RNA

<220>  
<221> misc\_feature  
<222> (1)..(17)

<400> 13  
gatcgttgg tcctgaa

17

<210> 14  
<211> 17  
<212> DNA  
<213> Artificial

## Giuliani ODN.ST25.txt

<220>  
<223> Antisense phosphorothioate oligonucleotide targeting the site  
298 of the human Smad 7 m-RNA

<220>  
<221> misc\_feature  
<222> (1)..(17)

<400> 14  
atcgtttgtt cctgaac

17

<210> 15  
<211> 21  
<212> DNA  
<213> Artificial

<220>  
<223> Modified antisense phosphorothioate oligonucleotide targeting the  
site 403 of the human Smad 7 m-RNA

<220>  
<221> misc\_feature  
<222> (1)..(1)  
<223> "n": 2'-deoxyguanosine methylphosphonate

<220>  
<221> modified\_base  
<222> (1)..(1)  
<223> 2'-deoxyguanosine methylphosphonate

<220>  
<221> misc\_feature  
<222> (3)..(3)  
<223> "n": 5-methyl 2'deoxycytidine 5' monophosphate

<220>  
<221> modified\_base  
<222> (3)..(3)  
<223> m5c

<220>  
<221> misc\_feature  
<222> (16)..(16)  
<223> "n": 5-methyl 2'deoxycytidine 5' monophosphate

<220>  
<221> modified\_base  
<222> (16)..(16)  
<223> m5c

<220>  
<221> misc\_feature  
<222> (20)..(20)  
<223> "n": 2'-deoxyguanosine methylphosphonate

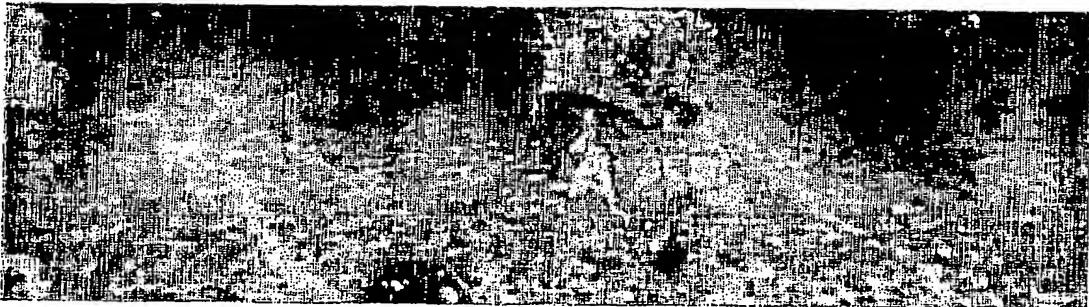
<220>  
<221> modified\_base  
<222> (20)..(20)  
<223> 2'-deoxyguanosine methylphosphonate

<400> 15  
ntngccccctt ctcccnngcan c

21

10/551643

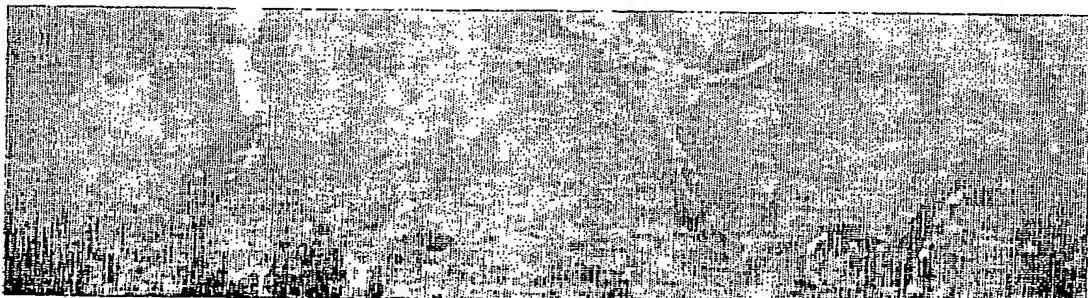
1/5



N.S.



ODN Sense



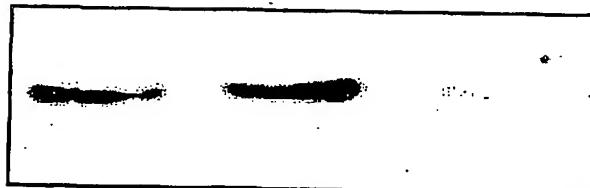
ODN Antisense

Fig. 1

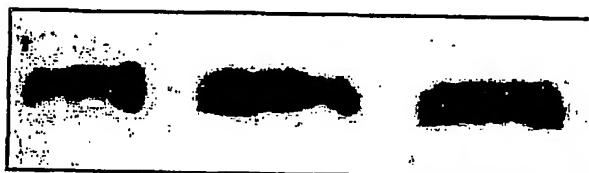
10/551643

2/5

Unt EtOH TNBS



p-Smad2/Smad3



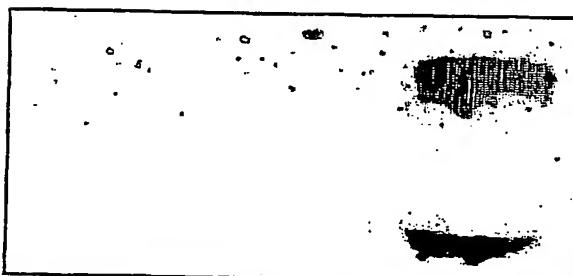
Smad2/Smad3

Fig. 2

Unt EtOH TNBS

116 KDa -  
85 KDa -

50 KDa -



Smad7



β-actine

Fig. 3

3/5

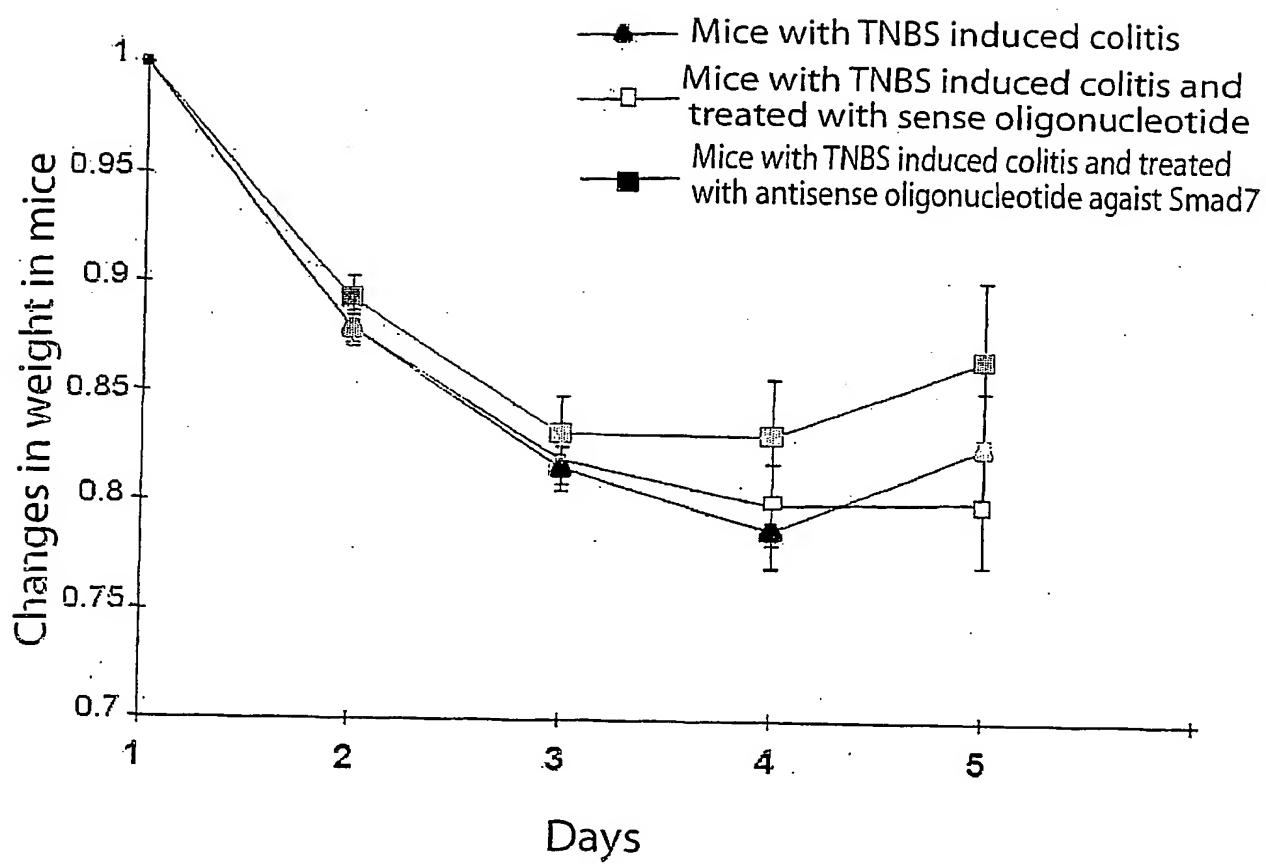


Fig. 4

10/551643

4/5

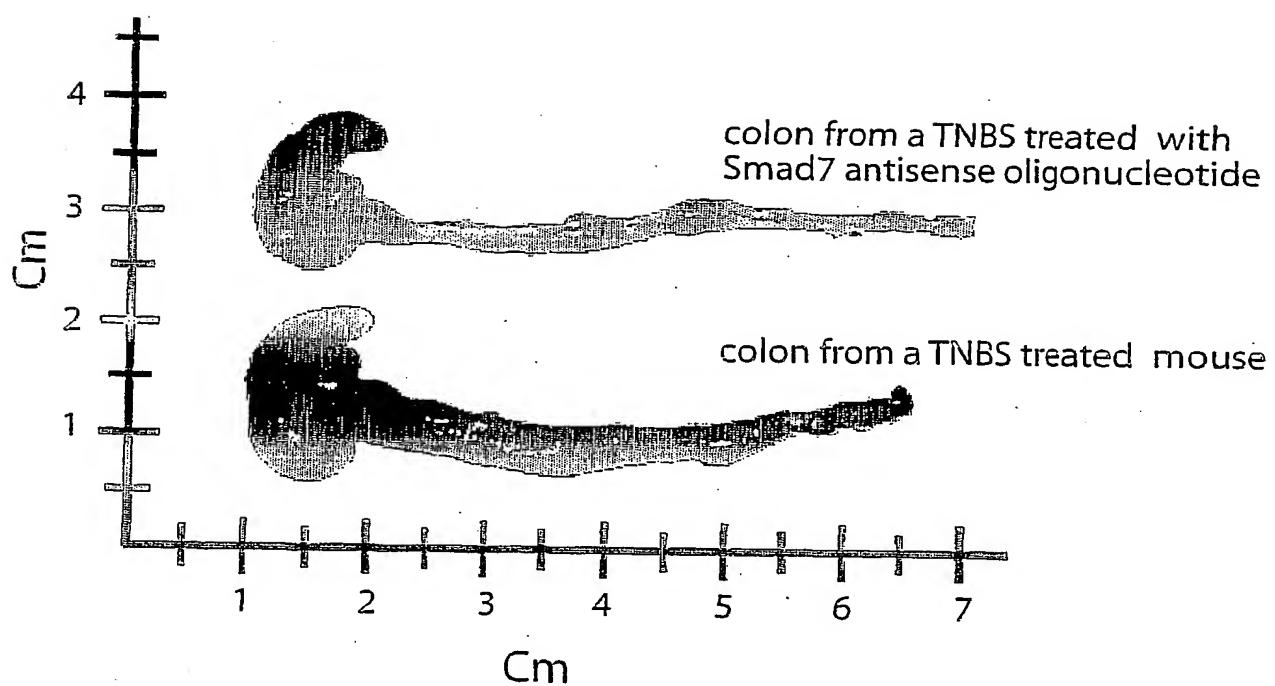
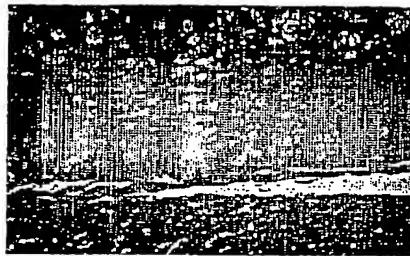


Fig. 5

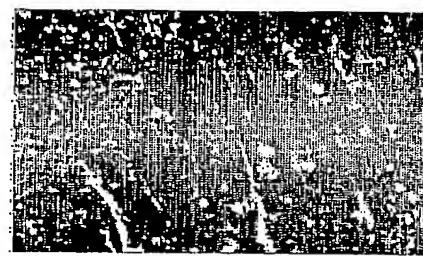
10/551643

5/5

Mice without colitis



Mice with TNBS induced colitis



Mice with TNBS induced colitis and  
treated with control oligonucleotide



Mice with TNBS induced colitis and  
treated with Smad7 oligonucleotide



Fig. 6